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### *Divergence of cryptic species of *Doryteuthis plei* Blainville, 1823 (Loliginidae, Cephalopoda) in the Western Atlantic Ocean is associated with the formation of the Caribbean Sea*

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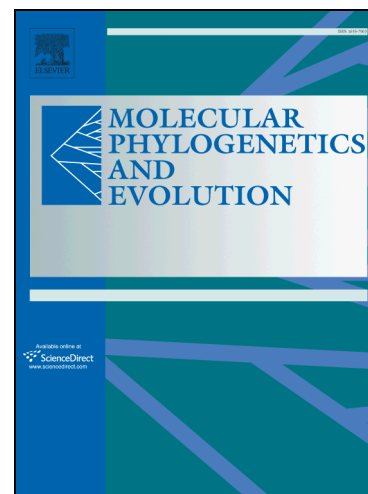
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## Editor's Choice Article

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**Divergence of cryptic species of *Doryteuthis plei* Blainville, 1823 (Loliginidae, Cephalopoda) in the Western Atlantic Ocean is associated with the formation of the Caribbean Sea.**

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## Abstract

Although recent years have seen an increase in genetic analyses that identify new species of cephalopods and phylogeographic patterns, the loliginid squid of South America remain one of the least studied groups. The suggestion that *Doryteuthis plei* may represent distinct lineages within its extensive distribution along the western Atlantic coasts from Cape Hatteras, USA (36°N) to northern Argentina (35°S) is consistent with significant variation in a number of environmental variables along this range including in both temperature and salinity. In the present study *D. plei* samples were obtained from a large number of localities along the western Atlantic coasts to investigate the distribution of these possible species in a phylogeographic context. Phylogeographic analyses were performed using the mitochondrial Cytochrome Oxidase I gene and nuclear Rhodopsin gene. Divergence times were estimated using Bayesian strict clock dating with calibrations based on fossil records for divergence from the lineage containing *Vampyroteuthis infernalis* (162 mya), the probable origins of the North American loliginids (45 mya), and the European loliginids (20 mya) and fossil statolith from *Doryteuthis opalescens* (3 mya). Our results suggest a deep genetic divergence within *Doryteuthis plei*. The currently described specie consists of two genetically distinct clades (pair-wise genetic divergence of between 7.7 and 9.1%). One clade composed of individuals collected in northwestern Atlantic and Central Caribbean Atlantic waters and the other from southwestern Atlantic waters. The divergence time and sampling locations suggest the speciation process at approximately 16 Mya, which is in full agreement with the middle Miocene orogeny of the Caribbean plate, ending up with the formation of the Lesser Antilles and the adjacent subduction zone, coinciding with a particularly low global sea level, resulting in the practical absence of continental shelves at the area, and therefore an effective geographic barrier for *D. plei*. Furthermore, this study also provides evidence of previously undocumented sub-population structuring in the Gulf of Mexico.

**Keywords:** *Phylogeography, COI, Rhodopsin, Doryteuthis plei, Speciation, Caribbean plate;*

## 1-Introduction

One of the greatest challenges for marine evolutionary biology is the diagnosis of the processes responsible for genetic differentiation of populations (Palpouline et al., 2004). Interactions between physical and biological factors such as those between ocean currents, benthic topology and dispersal capacity of different life stages of organisms (gametes, larvae, juveniles, adults) can result in different rates and patterns of gene flow (Weersing, et al., 2009; Hoffman, et al., 2013; Haye et al., 2014; Villamor, et al., 2014), and these in turn can greatly influence the evolutionary history of species (Hauser and Carvalho, 2008).

In theory, the presence of a planktonic life phase of marine organisms allows for a high dispersal capacity and can facilitate genetic homogeneity across the species' range (Palumbi, 1994). Evidence linking dispersion potential to reduced genetic differentiation has been found in various species (Avise, 1994). However, there are exceptions where high dispersal capacity does not result in genetic homogeneity (Gerlach et al., 2006; Teske et al., 2007). Considering these factors, invertebrates such as cephalopods that present specific biological characteristics that determine dispersal limits (association to substrates, dispersal time of different life stages, different salinity tolerances) may be important models that can be used to test hypothetical speciation processes. Cephalopods include both sessile species, and species capable of making large scale migrations. They may present internal or external fertilization, direct or indirect development and can be associated to a large range of different substrates (Roper et al., 1984; Nesis, 1987; Vecchione et al., 1998; Haimovici et al., 1994, Boyle and Rodhouse, 2005). Their distributions are often influenced by physio-chemical environmental factors that vary significantly at diverse spatial scales. These include salinity (Hendrix et al., 1981; Vecchione, 1991), ocean currents (Leite et al., 2008, Staaf et al., 2010) and the presence of specific substrates that are used for reproduction (Hanlon and Messenger, 1996; Boyle and Rodhouse, 2005).

Distinct species specific characteristics result in various patterns identified in population genetic studies of cephalopods in recent years. These include genetic homogeneity (Adcock et al., 1999; Shaw et al., 2004), isolation by distance between subpopulations (Perez-Losada et al., 2002), and reduction in gene flow related to

barriers (Shaw et al., 1999; Murphy et al., 2002). The majority of such studies involve species from northeastern Atlantic waters (Shaw et al., 1999; Murphy et al., 2002, Perez-Losada et al., 2002, Anderson et al. 2008), northwestern and western Atlantic species (Herke and Foltz, 2002; Buresch et al., 2006; Juárez et al., 2010), and, more recently, species from the north and southeastern Pacific (Reichow and Smith, 2001; Staaf et al., 2010; Ibanez et al., 2011; Sandoval-Castelanos et al., 2011; Ibanez et al., 2012) and indo-west Pacific (Yeatman and Benzie, 1993; Izuka et al., 1996; Anderson et al., 2011; Chen et al., 2014). The southwestern Atlantic remains one of the least studied regions (Adcock et al., 1999; Shaw et al., 2004; Moreira et al., 2011).

Loliginid squid are demersal, usually occupying coastal regions and limited to the continental shelf (to around 200 m depth), owing to a dependence on specific marine substrates for egg mass deposition, a characteristic considered to be important for delimiting the distribution of these species (Boyle and Rodhouse, 2005). Of the more economically and ecologically important species, *Doryteuthis plei* Blainville, 1823 is notable because of the presence of fisheries directed at this species in temperate regions of the distribution range (Jereb and Roper, 2010). Along with *Lolliguncula brevis* Steenstrup, 1881, this species displays the greatest geographic range for cephalopods along the western Atlantic coasts from Cape Hatteras, USA (36°N) to northern Argentina (35°S) (Jereb and Roper, 2010).

Phylogeographic inferences for populations of *D. plei* in the Gulf of Mexico and north-western Atlantic were made using the mitochondrial cytochrome oxidase I gene (COI), indicating the presence of sub-populations in these regions (Herke and Foltz, 2002). Furthermore, the potential for cryptic species in the genus *Doryteuthis* (*D. plei* and *D. pealei*) was suggested using mitochondrial and nuclear markers for small sample numbers from few sampling locations (Sales et al., 2013a). The present study uses both mitochondrial and nuclear markers to infer phylogeographic relationships of *D. plei* across its range to 1) determine whether *D. plei* is a single species presenting isolation by distance but with gene flow across its range or whether two, or more, cryptic species exist and 2) determine the distributional limits of any cryptic species identified and assess the potential for further sub-populations in the western Atlantic.

## 2-Material and Methods

### 2.1-Sampling and laboratory methods

A total of 169 specimens were collected for the present study from various localities between Campeche in Mexico (19°13'N, 91°02'W), to southern Santa Catarina state in Brazil (27°38'S, 48°13'W) (Figures 1 and 2, Supplementary data 1). A set of reference individuals were fixed in 10% formalin and deposited in the zoological collection of Oceanographic Museum at Federal University of Rio Grande (FURG). Additionally, 23 sequences from Herke and Foltz (2002) and three sequences from Sales et al. (2013a), were downloaded from Genbank and included in the final dataset (Supplementary data 1), resulting in a COI population analysis dataset containing sequences representing 195 individuals of *D. plei* covering the great majority of the distribution of the species (Jereb and Roper, 2010). All new specimens were identified using the taxonomic keys of Roper et al. (1984) and Jereb and Roper (2010). Procedures for preservation of tissues, DNA extraction, amplification by PCR and cycle sequencing are the same as in Sales et al. (2013a; 2014).

In addition to the new COI *D. plei* haplotypes, COI sequences were downloaded to produce a second dataset for analyses of divergence time including sequences for: *Loligo vulgaris* (Lamarck, 1798), *Loligo forbesi* (Steenstrup, 1856), *Loligo reynaudii* (Orbigny, 1839-1841), *Doryteuthis opalescens* (Berry, 1911), *Lolliguncula brevis* Blainville, 1823, *Lolliguncula diomedae* Steenstrup, 1881, *Sepioteuthis sepioidea* Blainville, 1824, *Ommastrephes bartramii* Lesueur, 1821; *Sthenoteuthis oalaniensis* Lesson, 1880 and *Vampyroteuthis infernalis* Chun, 1903 (Supplementary data 1). New rhodopsin haplotypes for *D. plei* were also obtained for various samples using amplification by PCR and cycle sequencing as in Sales et al. (2013a; 2014). (Supplementary data 1).

## 2.2-Molecular diversity analyses, genetic structuring and demographic history

Sequences were aligned using ClustalW (Thompson et al., 1997), implemented in BioEdit v.7.0.4 (Hall, 1999). Visual inspection was made after every alignment to correct possible errors in the automatic alignment process. The genetic diversity indices: Haplotype number (Hap); haplotype diversity ( $H$ ); and nucleotide diversity ( $\pi$ ) (Nei, 1987), were estimated for each sampling locality using Arlequin v. 3.11 (Excoffier et al., 2005).

The program Geneland (Guillot et al., 2005) implemented in R, was used to estimate similarity of populations across the sampling area, using multiple runs to infer the number of population groups (clusters) utilizing the COI + Rhodopsin database concatenated following these parameters: 5,000,000 Monte Carlo Markov Chain (MCMC) iterations, sampling every 5,000 iterations, and discard of the first 10% of samples as a “burn-in” phase. The best run was selected using the greatest mean posterior probability using the *post processing* function, and the “Map of population membership” and “Map of probability of population membership” produced based on populations identified under Hardy-Weinberg equilibrium with linkage equilibrium between loci (HWLE).

To confirm the clusters obtained in Geneland analyses a hierarchical analysis of molecular variance (AMOVA, Excoffier et al., 1992), was performed in Arlequin v. 3.11 (Excoffier et al., 2005). Genealogical relationships of haplotypes were obtained by creating a haplotype network in the program Haploviewer (Salzburger et al., 2011) using the Maximum Likelihood algorithm determined by jModelTest 2 (Darriba et al., 2012). The indices of  $F_s$  (Fu, 1997) and  $D$  (Tajima, 1989) and their respective  $P$  values were estimated in Arlequin v. 3.11 (Excoffier et al., 2005).

To analyze historical demography and divergence times, a molecular clock test was applied to the second sequence dataset (COI data - *D. plei* haplotypes and outgroups) to determine the best model in BEAST v. 1.7.4 (Drummond and Rambaut, 2007). Two simulations were made: 1-with a strict clock linked to mutation rate; 2- with relaxed clocks (log-normal and exponential). Simulations were compared using the Bayes factor (Suchard et al., 2001), as implemented in Tracer 1.5 (Rambaut and Drummond, 2009a), and the strict clock linked to mutation rate was determined to be more adequate (InBayesFactor=3.72). In this set of runs the mean rate selected for a



prior normal distribution was  $0.02 \times 10^{-6} \pm 0.005 \times 10^{-6}$  SD, the same mutation rate utilized in previous cephalopod studies (Pérez-Losada et al., 2007; Ibáñez et al., 2011; Ibáñez et al., 2012).

Because of the results of other analyses in this paper, the demographic history of *D. plei* populations was inferred using the COI data separated into two datasets, one including only samples from the north western Atlantic and one including only samples from the south western Atlantic. Both datasets were analyzed using the Bayesian Skyline Plot (BSP) in BEAST v. 1.7.4 (Drummond and Rambaut, 2007). The BSP analysis uses Monte Carlo Markov Chain (MCMC) methods based on nucleotide sequence data to estimate posterior distributions of the effective population size ( $N_e$ ) through time as well as Highest Posterior Density intervals (95% HPD). The BSP analysis was made using the evolutionary model selected by jModelTest 2 (Darriba et al., 2012), where for both datasets the Hasegawa-Kishino-Yano model with Gamma correction was selected ( $-\ln L = 1086.09$ ,  $BIC = 4401.90$ ) (HKY+G). Two independent MCMC runs were performed, each with 80,000,000 iterations and sampling every 1,000 generations. The first 8 million samples were discarded as “burn-in”. The program LogCombiner v. 1.7.4 (Rambaut and Drummond, 2009b) was used to combine the independent runs and produce the graphical BSP results.

### 2.3- Genetic divergence and estimates of divergence time

Genetic divergences were calculated using Kimura 2 parameters (K2P) distance model (Kimura, 1980) as implemented in MEGA v.6.0.6 (Tamura et al., 2013). To infer divergence time between the populations sampled in the present study, the MCMC method was implemented in BEAST v.1.7.4 (Drummond and Rambaut, 2007) using the same parameters established for BSP (see above). The HKY+G model and prior tree from the Yule process were used with the full complement of COI + Rodopsin haplotypes and sequences for closely related species downloaded from Genbank (the second dataset indicated in sampling).

The estimation of divergence times was made on BEAST v. 1.7.4 program (Drummond and Rambaut, 2007). Initially, the BEAST file was built in BEAUti

(Drummond *et al.*, 2007). The substitution model utilized was HKY (selected for AIC), with base frequencies estimated empirically, using the database partitioned by codon. All parameters were unlinked and mean substitution rate was unfixed. For the tree prior parameters, two simulations were performed. Initially, the tree prior Speciation: Yule Process was used. This simplest branching model assumes that, at any given point in time, every living lineage can speciate at the same rate,  $\lambda$ . Because the speciation rate is constant through time, there is an exponential waiting time between speciation events (Yule, 1924; Aldous, 2001), being more appropriate for species-level phylogenies (Drummond *et al.*, 2007). This model does not allow for extinctions. We also performed simulations utilizing the tree prior Birth-Death process, which is an extension of the Yule process. The Birth-Death model assumes that at any point in time every lineage can undergo speciation at rate  $\lambda$  or go extinct at rate  $\mu$  (Kendall, 1948; Rannala and Yang, 1996, Gernhard, 2008). These two simulations were run using a strict clock and the calibration nodes were constrained using lognormal-distribution priors.

The Time to Most Recent Common Ancestor (TMRCA) for the main clades was estimated using the following calibration points: A) 162 million years ago (Mya): appearance of the Order Vampyromorpha (Fischer and Riou, 2002; Strugnell *et al.*, 2006); B) 45 Mya: The probable origin of the North American loliginids (Clarke and Maddock, 1988); C) 20 Mya: The probable origin European loliginids; D) 3 Mya: Statolith fossils identified as belonging to *Doryteuthis opalescens* dated to the early Pleistocene (Clarke and Fitch, 1979). The MCMC method was used to infer divergence times between the northern and southern populations, run in BEAST 1.7.4 (Drummond and Rambaut, 2007). This was based on two independent MCMC runs of 160 million generations, with samples being taken every 1000 generations. The MCMC log files were combined in Tracer to summarize posterior divergences times with 95% highest posterior density limits; Only runs were the ESS values where greater than 200 for all marginal parameters were used, after discarding 10% of the first trees as burn-in.

Additionally, sea-level changes' reconstructions of Haq *et al.* (1988) and several different paleomaps of the Caribbean geologic evolution (Pindell and Barrett, 1990; MacMillan *et al.*, 2004; James, 2005; Miall and Blakey, 2008; Figueiredo *et al.*, 2009; Montes *et al.*, 2012) were uploaded in the SURFER v. 11.6 software (Golden Software, Inc.) and used to build up the geologic explanations for the reasons and time of divergence.

### 3-Results

#### 3.1-Phylogeographic Analyses

The final dataset of COI gene contained a complete 600 base pair sequence for all 195 individuals. For the Geneland analysis, the concatenated database (COI+Rhod) contained 1250 base pair. The phylogeographic structure determined by Geneland demonstrated a spatial differentiation of populations of *Doryteuthis plei* from the northwestern Atlantic and Gulf of Mexico in relation to individuals from the southwestern Atlantic (southwards from the Lesser Antilles) (Posterior probabilities > 0.4, Figure 1). Based on the population assignments of Geneland, the results of the AMOVA for COI gene corroborate the differentiation of these groups with greater differentiation between groups (northwestern Atlantic and Gulf of Mexico vs. southwestern Atlantic, 94.29%) compared to within groups (5.48%) ( $\Phi_{st}$ : 0.94339;  $p < 0.01$ , Table 1).

Similarly, the  $F_{ST}$  values obtained between populations from these regions were high and significant ranging from 0.924 (northwestern Atlantic vs. Espírito Santo) to 0.985 (Campeche vs. Amapá) (Table 2). Comparing  $F_{ST}$  values within these regions some differentiation is found between the northwestern Atlantic and Campeche (0.502-0.634), however, no indications of population structuring were found between these sampling locations in phylogenetic terms or in the Geneland analysis. By comparison little inter-sample population structure signal was found between localities along the southwestern Atlantic coastline ( $F_{ST}=0.096$ , Venezuela vs. Amapá;  $F_{ST}=0.058$ , Pará vs. Espírito Santo; and  $F_{ST}= 0.041$  Bahia vs. Espirito Santo) (Table 2). The AMOVA and diversity indices were also estimate for rhodospin haplotypes. For AMOVA, the same pattern observed in COI gene was recovered: The differentiations of groups are greater between Northwestern Atlantic/Gulf of Mexico vs. southwestern Atlantic, with 93.5% compared to within groups (3.28%) ( $\Phi_{st}$ : 0.96772;  $p < 0.01$ ) (Supplementary data 2). The  $F_{ST}$  values were significant (Supplementary data 3) between Northwestern Atlantic/Gulf of Mexico vs Southwestern Atlantic mainly when compared the Gulf of Mexico vs Southwestern Atlantic because the number of haplotypes used in our data base (Supplementary data 4).

The 91 haplotypes present in the haplotype network based on the evolutionary model TIM2+I (-lnL=1246.9533), show two clear clusters separated by 37 mutations between individuals from the northwestern Atlantic/ Gulf of Mexico and individuals from the southwestern Atlantic with no haplotypes shared between geographical regions (Figure 2). All sampled populations from the southwestern Atlantic shared haplotype 29, the most frequent haplotype in the region. No shared haplotypes were found in samples from the northwestern Atlantic and Gulf of Mexico, and haplotypes from Campeche formed a cohesive distinct unit within the northwestern Atlantic and Gulf of Mexico, with no apparent clustering of haplotypes to other populations (Figure 2).

### 3.2-Demographic Analyses

Considering all *D. plei* samples there was a high haplotype diversity ( $H=0.909$ ) and low nucleotide diversity ( $\pi=0.027$ ) (Table 3). Similar patterns are encountered within the northwestern Atlantic/ Gulf of Mexico and southwestern Atlantic populations, although nucleotide diversity is an order of magnitude lower ( $\pi=0.002$ - $0.006$ ), as well as within all sampling locations except for samples from Campeche ( $H=0.396$  and  $\pi=0.000$ ). Fu's  $F_s$  was negative and significant for all localities, indicating an excess of rare mutations compared to the number expected under neutral evolution and suggesting recent population expansion. These results are also supported by the Bayesian Skyline Plots, which show historical expansion of populations for both the northwestern Atlantic/Gulf of Mexico and the southwestern Atlantic; For the northwestern Atlantic/Gulf of Mexico, the effective population size increased from approximately 3,300,000 to approximately 13,900,000 individuals (HPD 95% 11,423,712 – 66,603,752 individuals) at last 70,000 years (Figure 3A). For the Southwestern populations, the effective population size increased for ~4,000,000 to 32,000,000 (HPD 95% 18,400,000 – 50,500,000 individuals) at last 60,000 years (Figure 3B).

### 3.3-Genetic divergence and divergence time estimates

Divergence values vary between 8.4% (*D. plei* from the northwestern Atlantic and Gulf of Mexico vs. *D. plei* from the southwestern Atlantic) to 23.8% (*D. plei* from

northwestern Atlantic and Gulf of Mexico vs. *D. sanpaulensis*) for COI data (Table 4) and from 0.8% (*D. plei* from the northwestern Atlantic and Gulf of Mexico vs. *D. plei* from the southwestern Atlantic) to 5.2% (*D. sanpaulensis* vs. *D. pealei* from the northwestern Atlantic) for Rhodopsin data (Table 5). Analyses of divergence time indicate that populations of *D. plei* from the northwestern Atlantic/Gulf of Mexico, and populations of *D. plei* from the southwestern Atlantic separated from each other around 16 Mya (95% HPD 9.857-22.254 Mya) (Figure 4).

## 4-Discussion

### 4.1-Phylogeographic inferences

The results of this study build on those of Sales et al. (2013a) which indicated possible cryptic speciation for *Doryteuthis plei* and *Doryteuthis pealei*, within the western Atlantic Ocean, by increasing spatial coverage and sample density across the known range of *D. plei*. The phylogeographic analyses performed here did not clearly support population differentiation among northwestern Atlantic and northern Gulf of Mexico populations of *D. plei*, but did indicate a possible subpopulation in the region of Campeche. No haplotypes were found to be shared by any of the populations. Neutrality tests and BSP indicate that these populations have likely undergone recent population expansion. Herke and Foltz (2002) had previously indicated the presence of at least two subpopulations of *D. plei* in the northern Gulf of Mexico. The presence of subpopulations in Loliginid squid has previously been associated with hydrographic and hydrodynamic barriers to migration between various coastal European and Azorean populations of *Loligo forbesi* (Shaw et al., 1999) and segregated inshore breeding populations of *Loligo (Doryteuthis) pealei* which mix in offshore overwintering grounds (Buresch et al., 2006). The latter of these two examples, which is from the same approximate geographic region, combined with the relatively lower capacity of DNA sequence data from moderate sample sizes to resolve subpopulations may explain why we did not recover the subpopulations indicated by Herke and Foltz (2002).

All analyses performed in this study strongly support substantial genetic divergence of *D. plei* samples from the northwestern Atlantic/Gulf of Mexico from samples from the southwestern Atlantic (COI gene K2P distance: 7.7-9.1%;  $\Phi_{st}$ :

0.94339;  $F_{ST}$ : 0.952,  $P < 0.01$ ), including analyses based on more slowly evolving rhodopsin nuclear gene (K2P distance: 0.8%). Similar divergence values for the equivalent gene sequences have been found to differentiate *Lolliguncula brevis* populations across the western Atlantic (6.5% for COI and 0.8% for Rhodopsin) and *Loligo reynaudii* from *Loligo vulgaris* between the northeastern and southeastern Atlantic (5.7% for COI and 0.9% for Rhodopsin) (Sales et al., 2014). As such, the divergence found between populations of *D. plei* from the northwestern Atlantic and southwestern Atlantic is comparable to species level divergence observed in other loliginid squid.

Comparing data for all southwestern Atlantic samples (between Isla Margarita, Venezuela and Santa Catarina, Brazil) there is a contrasting pattern to that observed for the northwestern Atlantic *D. plei* population, with all localities sharing the most frequently observed haplotype (haplotype 25), indicating that there are no barriers to dispersal and gene flow between these populations along the South American Atlantic coast, or that recent population expansion and dispersal along the entire coast has occurred. The latter is supported by the BSP analyses and neutrality tests. This pattern of haplotype dominance has been found previously in other squid species (Sandoval-Castellanos et al., 2010; Ibáñez et al., 2011; Ibáñez et al., 2012).

Despite indications of historical population expansions *D. plei* exhibits slightly higher mtDNA diversity (both haplotype and nucleotide diversity) than observed for many other cephalopods, and may reflect reduced effects of Pleistocene glacial cycles on population sizes and distributions in this warm temperate / sub-tropical species compared to other abundant and neritic colder water species (see Ibanez et al. 2011, 2012; Strugnell et al. 2012). Higher haplotype diversity ( $H=0.96-1.00$ ) in the northern compared to southern ( $H=0.71-0.98$ ) population may reflect larger and historically more stable population sizes in the northern population, the latter being supported by the BSP plots, although it may also reflect sampling effects from the smaller sample sizes from the northern region. Different population responses in squid to climate-induced environmental changes (such as during Pleistocene glacial cycles) have been observed in other species with wide latitudinal distributions (Ibanez et al. 2012), and large habitat variation across the range may also influence maintenance of standing genetic variation (see section 4.2).



#### 4.2-Divergence time and oceanographic barriers

The originally described distribution for *Doryteuthis plei* is for continental shelf habitats from Cape Hatteras (36°N) to northern Argentina (35°S), including the Gulf of Mexico, Caribbean Sea, Caribbean island chain and Bermuda (Jereb and Roper, 2010). There is significant variation in a number of environmental variables along this range including in both temperature and salinity, the latter of which is particularly influenced by outflow of major rivers such as the Amazon plume (Oltman, 1968; Muller-Karger et al., 1988) that may act as a barrier to dispersal of both adults (that reproduce in more saline environments) as well as paralarvae that show high mortality in low salinity environments (O'Dor and Weber, 1986; Vecchione, 1991; Hanlon and Messenger, 1996; Hanlon, 1998; Boyle and Rodhouse, 2005). The Amazon discharges approximately 6,300 km<sup>3</sup>year<sup>-1</sup> (Milliman and Meade, 1983), with a freshwater plume extending about 200 km from the Amazon River mouth, and reaching the 30 m isobath along the medium portion of the continental shelf (Curtin and Legeckis, 1986; Nitttrouer and DeMaster, 1996; Masson and Delecluse, 2001). However, below 30 m (and sometimes less) more typical marine saline conditions and currents prevail (Lumpkin and Garzoli, 2005; Salisbury et al., 2011; Moura et al., 2016). The recent study of Moura et al. (2016) also confirmed that at the Amazon River mouth area, between depths of 30 to 100 m, the Amazon plume is rather thin and more typical marine saline conditions prevail beneath the plume, where even mesophotic reefs occur and would characterize a biodiversity corridor across the Amazon River mouth. Loliginid squid are known to be capable of making considerable horizontal and vertical migrations (Nesis 1987; Boyle and Rodhouse, 2005; Jereb and Roper, 2010) which would potentially reduce the effect of the current outflow as a barrier. This is confirmed by the present study, the southwestern Atlantic samples come from locations both to the south (various locations) and north (Isla Margarita, Venezuela - 11°00'37"N, 63°58'44"W and Amapá, Brazil - 03°04'61"N, 49°32'32"W) of the current Amazon plume and are all genetically similar.

With the sample coverage in this study it was possible to identify that the *D. plei* populations from the northwestern Atlantic and southwestern Atlantic (suggested to be different species based by the current work) are geographically separated either side of

the Caribbean, substantially to the north of the current outflow of the Amazon, and that this division occurred approximately 16 Mya. Between the early and middle Miocene (20 – 10 Mya) the orogeny of the western Andes mobilized the Caribbean blocks converting the previous transform fault between the Caribbean plate and South American plate into a convergent fault, resulting in orogeny and intense volcanism that ended up forming the Lesser Antilles volcanic island chain and adjacent subduction zone (Pindell and Barrett, 1990; James, 2005). Between 11 and 10 Mya, there were significant changes in global sea level, lowering 70 m in relation to current sea level and more than 100 m in relation to the previously existing sea level (Haq et al., 1988). This was caused by the gradual migration of the Antarctic continent to its current position at the South Pole, which resulted in formation of the Antarctic polar ice sheet and consequent lowering of the sea level (Haq et al., 1988).

These two factors together would have drastically reduced the continental shelf area around the Lesser Antilles (Haq et al., 1988; Pindell and Barrett, 1990; James, 2005). Besides, the late Miocene Caribbean evolution resulted in the establishment of the Anegada Passage, in association with the Muertos Trench, forming a deep water natural barrier for shallow water organisms as *D. plei*. Furthermore, at this time, oceanic currents were different and the Amazon River did not drain into the Atlantic at its current location as the establishment of the Amazon as a transcontinental river occurred about 10 Mya (Figueiredo et al., 2009). Figure 5 summarizes the main aforementioned geologic events and features that built up a natural barrier, based on different sources of information (Haq et al., 1988; Pindell and Barrett, 1990; MacMillan et al., 2004; James, 2005; Miall and Blakey, 2008; Figueiredo et al., 2009; Montes et al., 2012).

Oceanic currents are reported to have generally flown westwards across northern South America through the Isthmus of Panama region until the closure of this path 2.6 Mya (Nesbitt and Young, 1997). The onset of the Nicaragua rise establishing the Caribbean-Loop current system to the north (15-12 Mya) and strengthening of the Gulf Stream (Droxler et al., 1998) may have allowed for expansion/colonization between North and South during the earliest phases of the formation of the Caribbean. Subsequent changes in currents between 12 Mya and 2.6 Mya related to sea-level changes and connectivity between the Atlantic and Pacific may then have kept



populations on the South American and North American continents isolated, allowing genetic divergence.

Between the early and late Miocene the drainage patterns in northern South America also changed significantly. Originally much of northwest Amazonia drained northward along the paleo–Orinoco river system to a delta near the present day Lake Maracaibo. In the late middle Miocene uplift of the Eastern Cordillera resulted in development of the Amazon, but drainage fed and increased outflow of the paleo–Orinoco river system toward the Caribbean. Finally, only in the late Miocene the substantial Andean uplift caused the Orinoco and the Amazon to change their courses to the Atlantic where they are now (Hoorn et al., 1995; Figueiredo et al., 2009). It is still unclear how much freshwater would have made it into the Caribbean and how this may or may not have influenced salinity, particularly along a much reduced continental shelf resulting from a drop in sea level. However, the possibility of reduced salinity influencing the fauna of the region cannot be completely ignored.

The distributions of cephalopod species are determined by salinity, ocean currents and the presence of substrates for egg deposition (Hanlon and Messenger, 1996; Norris, 2000; Rocha et al., 2001, Boyle and Rodhouse, 2005, Rodrigues and Gasalla, 2008). As such, any one of the factors listed above or, more likely, a combination of them in this region may have reduced gene flow between populations of *D. plei* from the northwestern Atlantic and southwestern Atlantic around the period at which they are estimated to have diverged. Species of *Doryteuthis* are limited to depths of up to 370 m, occupying deeper waters during the day and performing vertical migrations to feed and reproduce at night. The presence of relatively firm substrates is extremely important for these species as deposition sites where egg masses are laid to develop before hatching into paralarvae (Hanlon and Messenger, 1996; Shaw and Boyle, 1997, Buresch et al., 2006, Moreira et al., 2011).

Continued orogeny of the Antilles and frequent changes in sea level associated with glaciations in the Pleistocene may have helped maintain the separation of these populations allowing the fixation of genetic differences for what are now suggested to be distinct species. The role of currents may be important for these species, as well as other cephalopods (Shaw et al., 1999; Leite et al., 2008; Staaf et al., 2010, Sales et al., 2013b), as indicated by the presence of subpopulation structuring when comparing *D.*

*plei* from Campeche with other locations from the northwestern Atlantic and Gulf of Mexico. The Yucatán current flows north from the Caribbean into the Gulf of Mexico via the Yucatán Canal and then flows westwards causing a series of eddies in the western Gulf of Mexico (Martínez and Pares, 1998; Juárez et al., 2010). Larval dispersal can therefore be limited to specific areas and result in divergence between populations, impeding larval transport from the Gulf of Mexico southwards, and tending to reduce connectivity between populations in Campeche from populations in the northern Gulf of Mexico.

The present study provides evidence that *Doryteuthis plei* from the northwestern Atlantic and Gulf of Mexico represents a genetically distinct species from *D. plei* in the southwestern Atlantic, where oceanographic phenomena and tectonic changes associated with the formation of the Caribbean Sea are likely to have influenced the speciation process approximately 16 Mya. The type locality for *Doryteuthis plei* is the island of Martinique in the Lesser Antilles, close to our sampling locality (Isla Margarita, Venezuela). Therefore, as in the case of *Lolliguncula brevis* (Sales et al., 2014) the new species that requires official description is that from the northern part of the range. As well as a new example of cryptic species in cephalopods, this study also provides evidence of previously undocumented sub-population structuring in the Gulf of Mexico.

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### Sales et al., *Doryteuthis* 2016. Table and Figure Legends:

Table 1: Values of *Fst* and AMOVA of populations *Doryteuthis plei* used in this study. **na**: North Atlantic; **gme**: Gulf of Mexico (East); **gmw**: Gulf of Mexico (West); **camp**: Campeche; **ve**: Venezuela; **ap**: Amapá state; **pa**: Pará state; **pb**: Paraíba state; **ba**: Bahia state; **es**: Espírito Santo state; **rj**: Rio de Janeiro state; **pr**: Paraná state; **sc**: Santa Catarina state;

Table 2: Indices of genetic differentiation (*Fst*, lower diagonal) based on the COI gene, obtained for populations of *Doryteuthis plei* used in this study. **NA**=America do Norte; **GME**: Leste do Golfo do México; **GMW**: Oeste do Golfo do México; **Camp**: Campeche; **VE**: Venezuela; **AP**: Amapá; **PA**: Pará; **PB**: Paraíba; **BA**: Bahia; **ES**: Espírito Santo; **RJ**: Rio de Janeiro; **PR**: Paraná; **SC**: Santa Catarina.

Table 3: Indices of genetic diversity obtained for the COI gene populations *Doryteuthis plei* used in this study. Bold values were significant. N = Number of subjects; Hap = number of haplotypes; (H) = haplotype diversity. ( $\pi$ ) = nucleotide diversity;

Table 4: Genetic distances estimated of cytochrome *c* oxidase I gene (COI) of the *Doryteuthis* species utilized in the present study.

Table 5: Genetic distances estimated of rhodopsin gene (Rhod) of the *Doryteuthis* species utilized in the present study.

Supplementary Data 1: Code used and sampling location of *Doryteuthis plei* samples used in this study. The letters in brackets represent the population chosen for dividing the samples into the Haploviewer program. Samples in bold were used for divergences times estimation inferences.

Supplementary Data 2: Values of *Fst* and AMOVA of populations *Doryteuthis plei* based on the rhodopsin gene used in this study. **na**: North Atlantic; **gmw**: Gulf of Mexico (West); **camp**: Campeche; **ve**: Venezuela; **ap**: Amapá state; **pa**: Pará state; **pb**: Paraíba state; **ba**: Bahia state; **es**: Espírito Santo state; **rj**: Rio de Janeiro state; **pr**: Paraná state; **sc**: Santa Catarina state;

Supplementary Data 3: Indices of genetic diversity obtained for the rhodopsin gene for populations of *Doryteuthis plei* used in this study. Bold values were significant. N = Number of subjects; Hap = number of haplotypes; (H) = haplotype diversity. ( $\pi$ ) = nucleotide diversity;

Supplementary Data 4: Indices of genetic differentiation ( $F_{st}$ , lower diagonal) based on the rhodopsin gene, obtained for populations of *Doryteuthis plei* used in this study. **NA**=America do Norte; **GMW**: Oeste do Golfo do México; **Camp**: Campeche; **VE**: Venezuela; **AP**: Amapá; **PA**: Pará; **PB**: Paraíba; **BA**: Bahia; **ES**: Espírito Santo; **RJ**: Rio de Janeiro; **PR**: Paraná; **SC**: Santa Catarina;

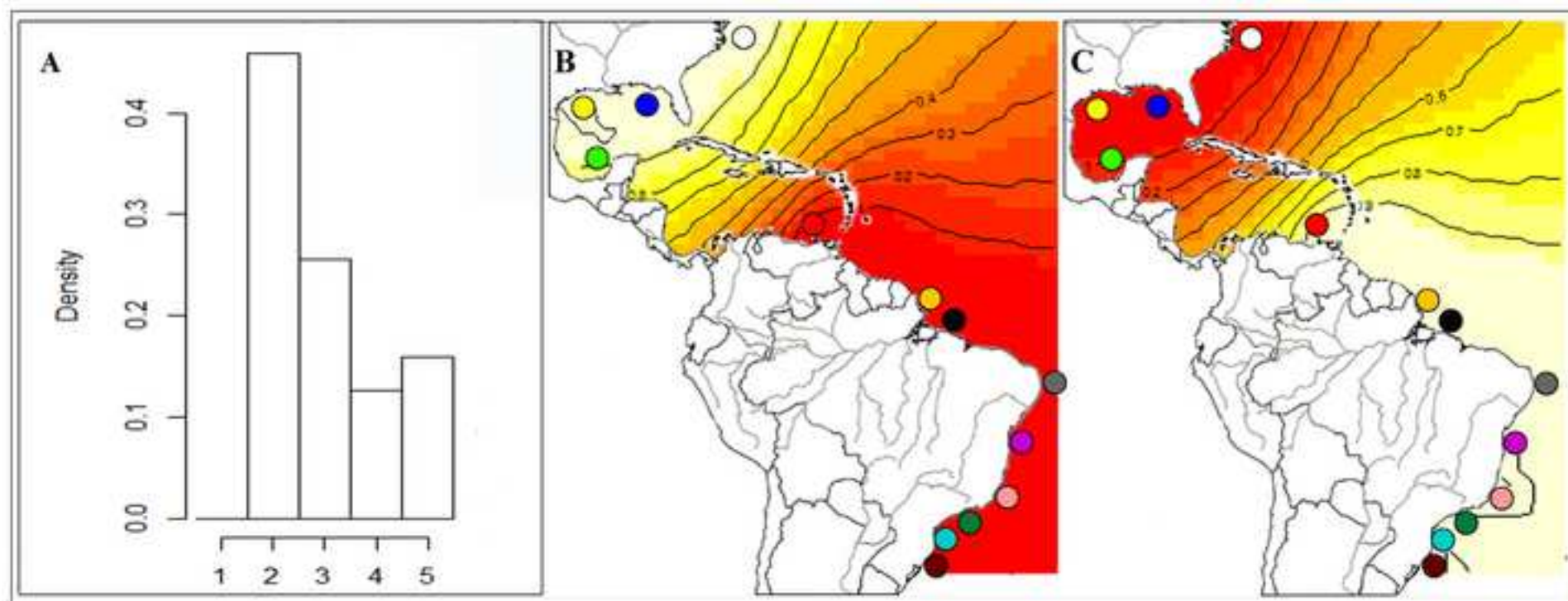
Figure 1: Geneland HWLE population maps A: Map of Posterior probability of *Doryteuthis plei* populations representing the number of clusters after the burnin. B: Spatial posterior probability of belonging to south-western Atlantic *D. plei* populations; C: Spatial posterior probability of belonging to north-western Atlantic *D. plei* populations;

Figure 2: Haplotype network of mtDNA COI sequence data using the Maximum Likelihood method and the evolutionary model TIM2+I. Colors correspond to the same locations shown in Figure 1. The amount of haplotypes in each circle is indicated by the representation. The number in the center of the haplotype network correspond to mutations between the North/Central and South specimens.

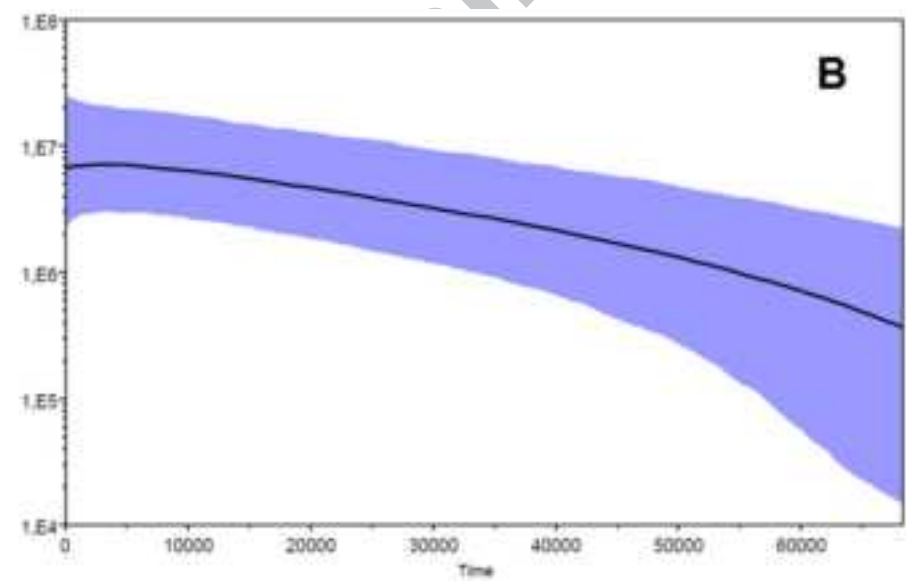
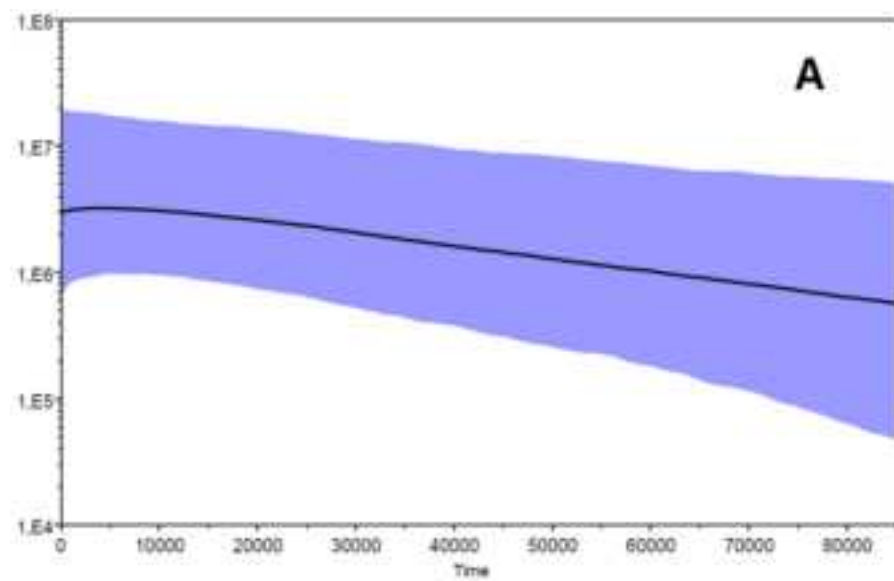
Figure 3: Bayesian Skyline Plot of *Doryteuthis plei* from Western Atlantic Ocean of COI gene. In figure A, all individuals from North/Central Atlantic Ocean (NA, GME, GMW and Camp). In figure B, all individuals from Southern Atlantic Ocean (VE, AP, PA, PB, BA, ES, RJ, PR and SC). Population size on the y-axis is given on a logarithmic scale. Time on the x-axis is given in years. The thick solid line represents the mean estimate of population size; the blue area shows the 95% highest posterior density intervals.

Figure 4: Bayesian strict-clock cladogram based on COI+Rhod haplotypes with dates of divergence between taxa: A=*Vampiroteuthis infernalis* vs. others: 157.39 mya (137.95 – 176.19 mya); B= The probable origin of the North American loliginids: 45.04 mya (44.04 - 45.96 mya); C= The probable origin European loliginids: 20.06 mya (19.06- 20.99 mya); D=*Doryteuthis opalencensis*: 2.44 mya (1.40-3.46 mya) The Star correspond to the splitting time between *Doryteuthis plei* from North/Central and South America 15.86 mya (9.85-22.25 mya);

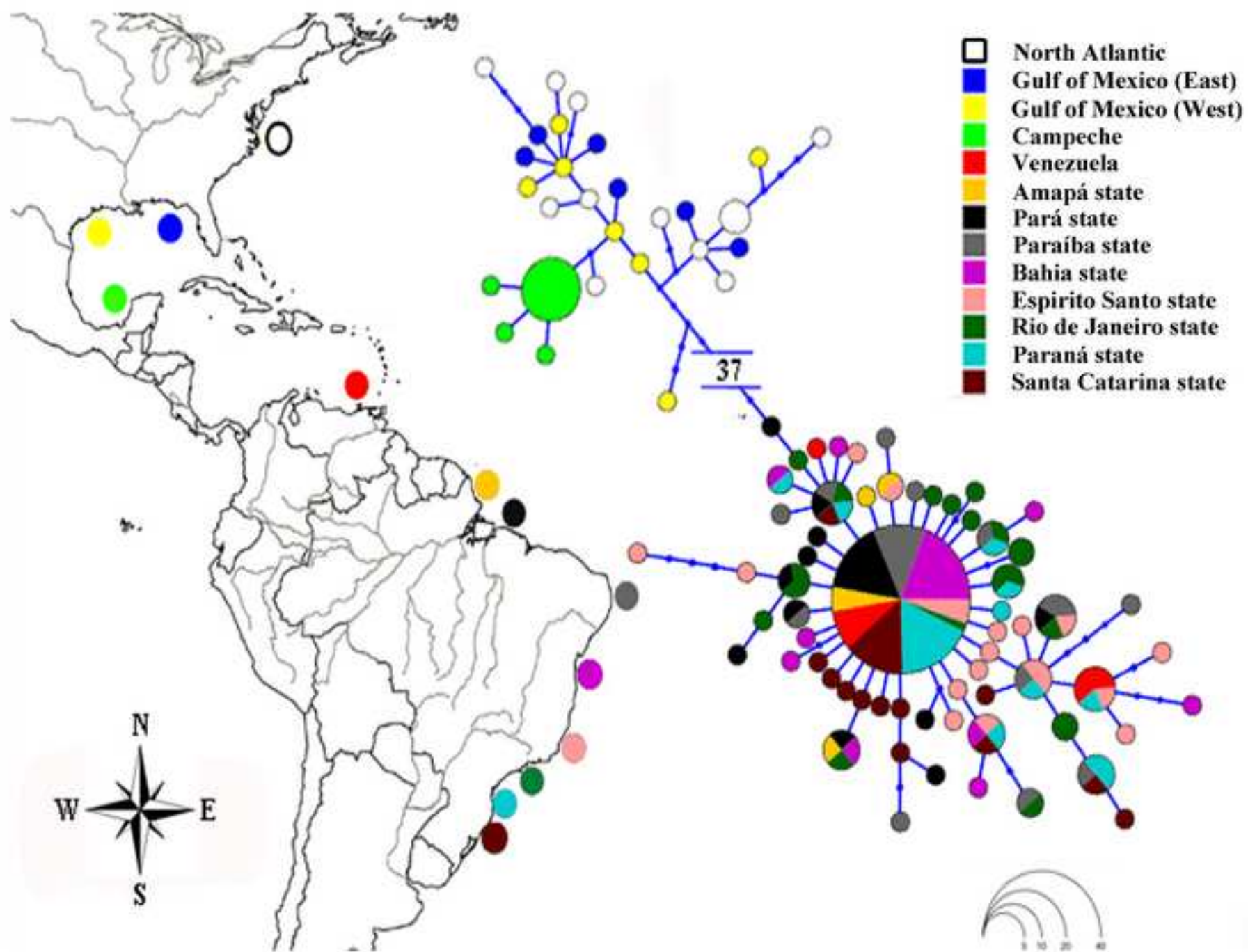
Figure 5: Geologic configuration and main events and features (blue lines) that result in the almost absence of shallow waters (e.g. continental shelf) between Bahamas and South America. Red line represents the present coastline; Thick black line the general shelf break, and the green line the former sea level (modified from Pindell and Barrett, 1990, with additional information from Haq et al., 1988; MacMillan et al., 2004; James, 2005; Miall and Blakey, 2008; Figueiredo et al., 2009; Montes et al., 2012).

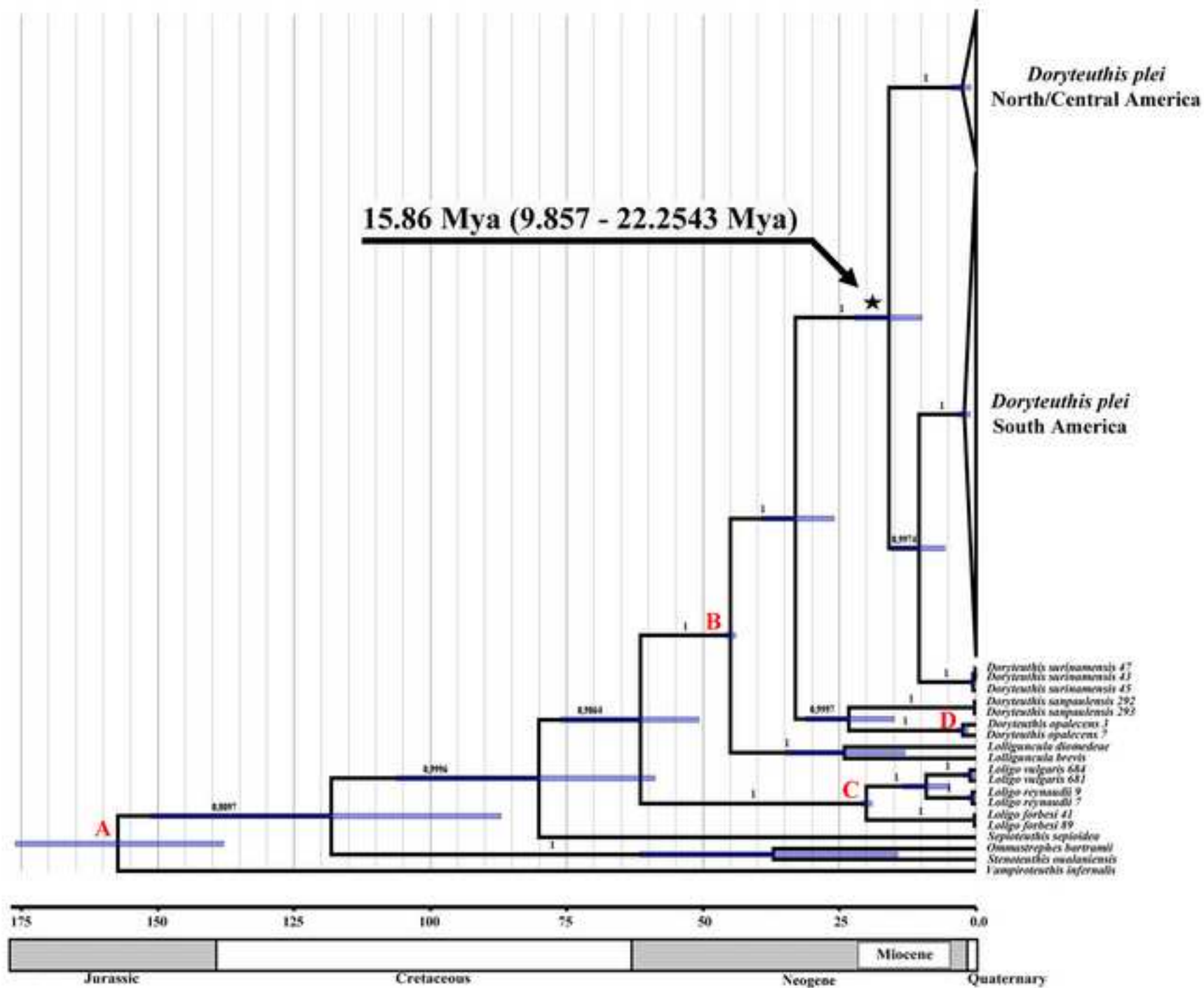


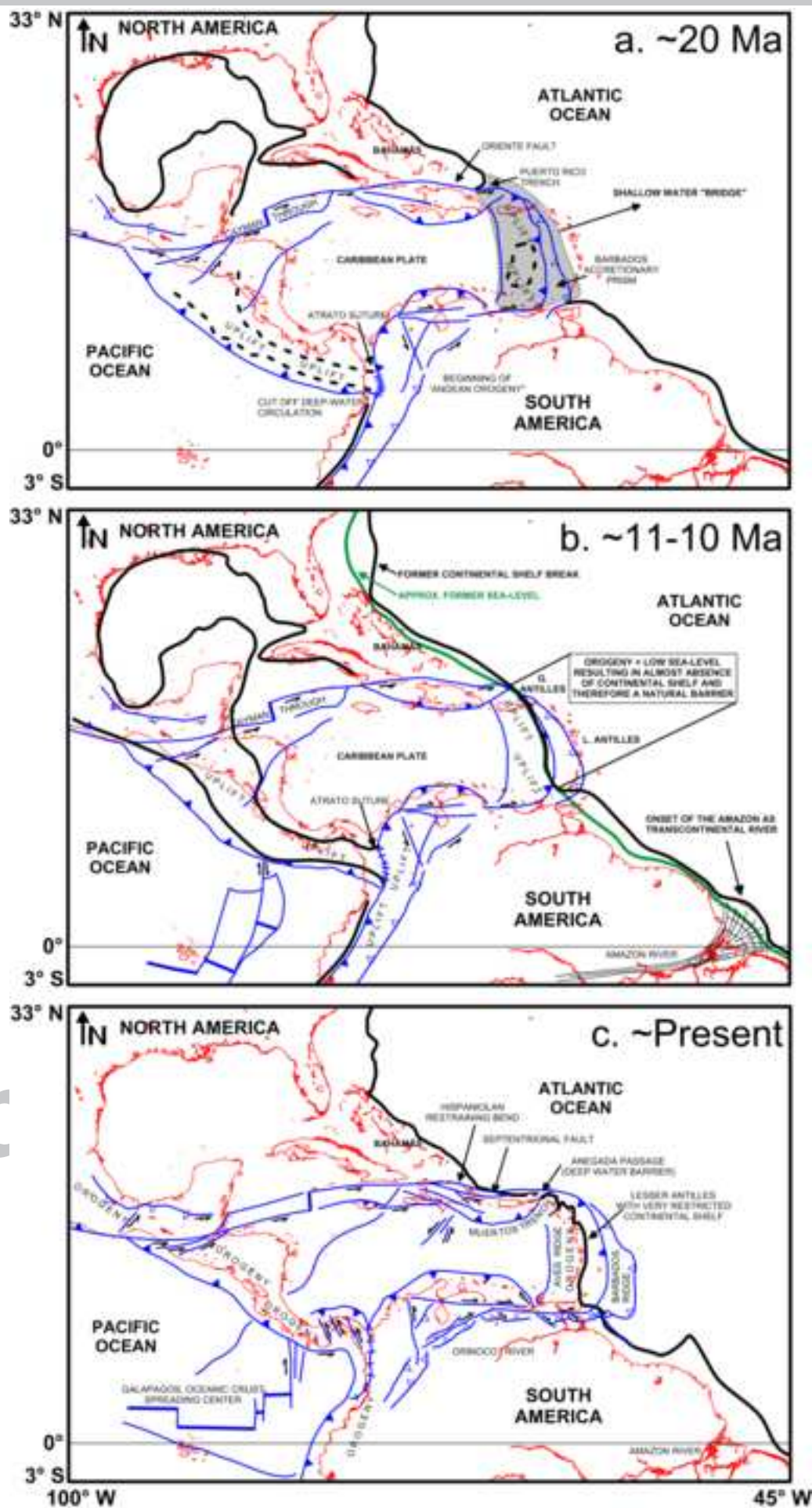












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*Doryteuthis plei*

*North and Central Atlantic (North America; Gulf of Mexico East; Gulf of Mexico West; Campeche) x South Atlantic (Venezuela; Amapa; Para; Paraíba; Bahia; Espirito Santo; Rio de Janeiro; Parana; Santa Catarina).*

Source of variation	% of Variation	$\Phi_{st}$
Between Populations	94.29	0.94339*
Among populations within groups	0.23	
Within Populations	5.48	

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\*Significance value ( $P < 0.01$ )

	NA	GME	GMW	Camp	VE	AP	PA	PB	BA	ES	RJ	PR	SC
NA	0,000	-	-	-	-	-	-	-	-	-	-	-	-
GME	-0,025	0,000	-	-	-	-	-	-	-	-	-	-	-
GMW	-0,024	0,029	0,000	-	-	-	-	-	-	-	-	-	-
Camp	0,502*	0,634*	0,543*	0,000	-	-	-	-	-	-	-	-	-
VE	0,931**	0,946**	0,946**	0,979**	0,000	-	-	-	-	-	-	-	-
AP	0,930**	0,947**	0,948**	0,985**	0,096*	0,000	-	-	-	-	-	-	-
PA	0,941**	0,952**	0,952**	0,973**	0,070	-0,042	0,000	-	-	-	-	-	-
PB	0,928**	0,934**	0,935**	0,960**	-0,002	-0,012	0,005	0,000	-	-	-	-	-
BA	0,938**	0,948**	0,948**	0,970**	0,037	-0,041	-0,012	0,005	0,000	-	-	-	-
ES	0,924**	0,929**	0,930**	0,956**	-0,050	0,035	0,058*	0,000	0,041*	0,000	-	-	-
RJ	0,925**	0,930**	0,931**	0,957**	0,022	-0,022	0,002	-0,014	0,007	0,029	0,000	-	-
PR	0,941**	0,952**	0,952**	0,973**	0,004	0,004	0,003	0,022	-0,009	0,006	-0,022	0,000	-
SC	0,938**	0,947**	0,947**	0,969**	0,032	-0,027	-0,011	-0,014	-0,007	0,029	-0,014	-0,027	0,000

\*Significance value (P<0.05); \*\* Significance value (P<0.01); Sampling points abbreviation are: NA (North America), GME (Gulf of Mexico East), GMW (Gulf of Mexico West), Camp (Campeche), VE (Venezuela), AP (Amapá), PA (Pará), PB (Paraíba), BA (Bahia), ES (Espírito Santo), RJ (Rio de Janeiro), PR (Paraná) and SC (Santa Catarina);

Population	N	Hap	( <i>H</i> ) (sd)	( $\pi$ ) (sd)	<i>D</i> Tajima	<i>F<sub>s</sub></i> <i>F<sub>u</sub></i>
NA	13	11	0.962 (0.050)	0.006 (0.001)	-1.673	<b>-5.702**</b>
GME	6	6	1.000 (0.096)	0.005 (0.000)	-0.286	<b>-3.032*</b>
GMW	7	7	1.000 (0.076)	0.005 (0.000)	-0.197	<b>-4.266*</b>
Camp	14	4	0.396 (0.159)	0.000 (0.000)	-1.670	<b>-2.288*</b>
AP	6	4	0.800 (0.172)	0.002 (0.001)	-1.295	<b>-1.252*</b>
PA	20	12	0.811 (0.092)	0.002 (0.001)	<b>-2.005*</b>	<b>-8.577**</b>
PB	20	13	0.884 (0.067)	0.004 (0.001)	<b>-1.901*</b>	<b>-7.172**</b>
BA	20	10	0.711 (0.113)	0.003 (0.001)	<b>-2.042*</b>	<b>-4.524*</b>
ES	20	17	0.979 (0.024)	0.005 (0.001)	<b>-1.563*</b>	<b>-14.832**</b>
RJ	20	16	0.979 (0.021)	0.005 (0.001)	-1.506	<b>-12.619**</b>
PR	20	10	0.758 (0.101)	0.002 (0.001)	-1.270	<b>-5.162**</b>
SC	20	13	0.853 (0.083)	0.003 (0.001)	<b>-1.569*</b>	<b>-9.384**</b>
<b>Total</b>	195	91	0.909 (0.018)	0.027 (0.002)	-1.382	<b>-38.742*</b>

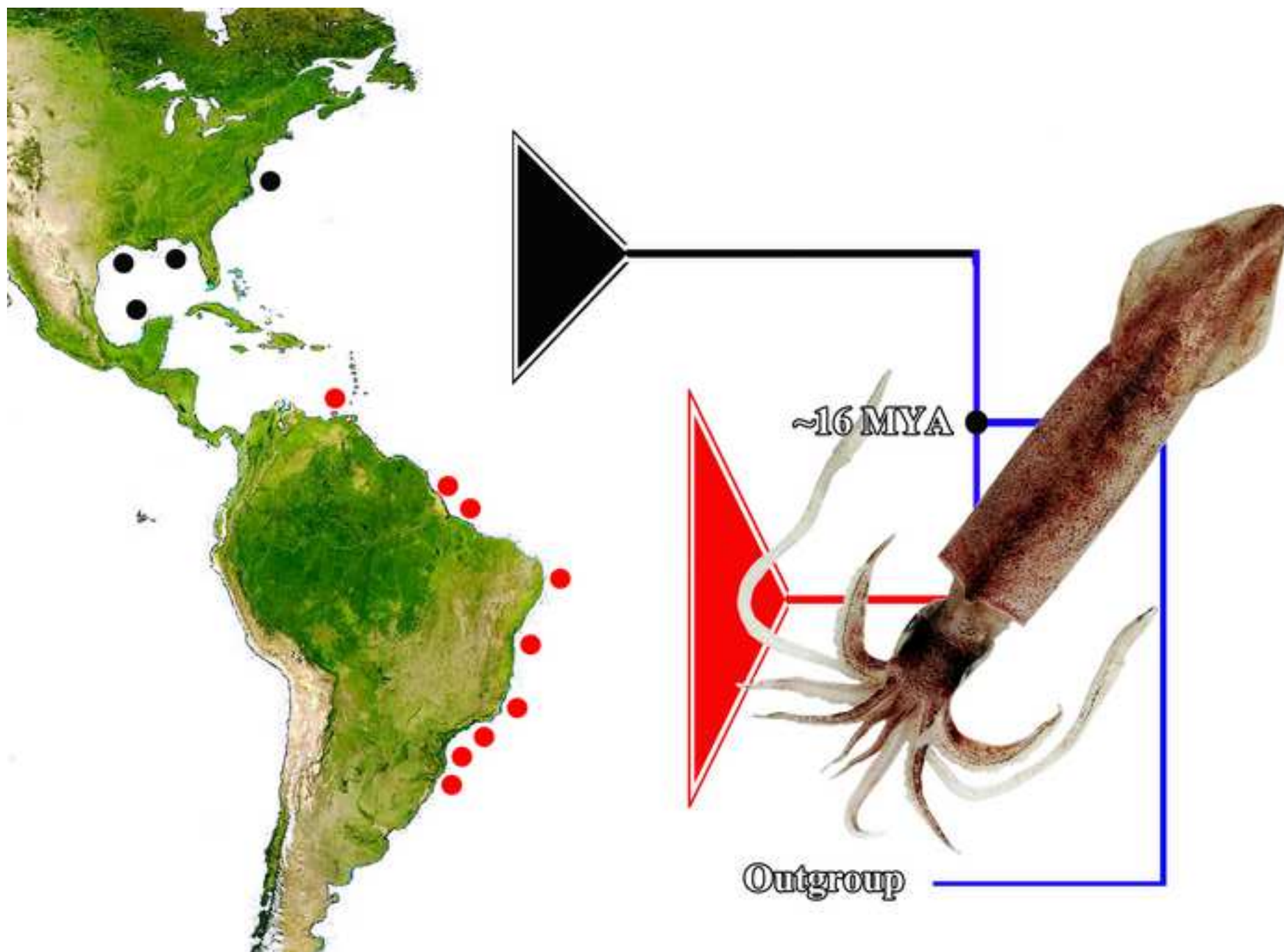
\*Significance Value ( $P < 0.05$ ); \*\* Significance Value ( $P < 0.01$ ); Sampling points abbreviation are: NA (North America), GME (Gulf of Mexico East), GMW (Gulf of Mexico West), Camp (Campeche), VE (Venezuela), AP (Amapá), PA (Pará), PB (Paraíba), BA (Bahia), ES (Espírito Santo), RJ (Rio de Janeiro), PR (Paraná) and SC (Santa Catarina);



COI	<i>D. plei</i> North/Central	<i>D. plei</i> South	<i>D. pealei</i> North	<i>D. sanpaulensis</i>	<i>D. gahi</i>
<i>D. plei</i> North/Central	-				
<i>D. plei</i> South	0.084	-	-	-	-
<i>D. pealei</i> North	0.174	0.170	-	-	-
<i>D. sanpaulensis</i>	0.238	0.207	0.188	-	-
<i>D. gahi</i>	0.191	0.179	0.157	0.194	-
<i>D. opalescens</i>	0.192	0.181	0.159	0.199	0.190

Rhod	<i>D. plei</i> North/Central	<i>D. plei</i> South	<i>D. pealei</i> North	<i>D. sanpaulensis</i>	<i>D. gahi</i>
<i>D. plei</i> North/Central	-	-	-	-	-
<i>D. plei</i> South	0.008	-	-	-	-
<i>D. pealei</i> North	0.042	0.035	-	-	-
<i>D. sanpaulensis</i>	0.034	0.037	0.052	-	-
<i>D. gahi</i>	0.040	0.040	0.048	0.042	-
<i>D. opalescens</i>	0.030	0.030	0.043	0.024	0.038





### Highlights

- 1-We analyzed the phylogeographical of *D. plei* in Atlantic coasts of the Americas;
- 2-COI and Rhod data reveal that *D. plei* are distinct between N and S American coasts.
- 3-Subpopulations were found in the North/Central Atlantic Ocean.
- 5-Caribbean orogeny and low sea level at Miocene provided a barrier.